Reference: *Biol. Bull.* **168**: 249–262. (April, 1985)

HOLOTHURIAN OOCYTE MATURATION INDUCED BY RADIAL NERVE[1]

YOSHIHIKO K. MARUYAMA

Department of Zoology, Faculty of Science, Kyoto University, Sakyo-Ku, Kyoto 606, Japan

ABSTRACT

Endogeneous substances responsible for maturation of holothurian oocytes were examined. Water-extracts of radial nerves from five species of sea cucumbers induced oocyte maturation. Cross-experiments on the maturation-inducing activity of radial nerve extracts indicated that the radial nerve extracts cross-react effectively among the sea cucumbers examined. From its heat-stability, protease-sensitivity, dialyzability, and elution-patterns through 'Sephadex' G-15 and G-50 columns, the active factor appears to be a heat-stable peptide of several thousands' daltons. The active factor, termed radial nerve factor, acted on oocytes in isolated ovaries or isolated oocytes having follicle cells to induce maturation. It was ineffective on oocytes deprived of follicle cells. Isolated ovaries or testes incubated with radial nerve factor produced a secondary factor which directly induces maturation of the follicle cell-free oocyte. Follicle cells isolated from follicle-oocyte complexes also produced the secondary factor in the presence of radial nerve factor. These results show that the radial nerve factor stimulates the follicle cells to produce a secondary factor, and the latter, in turn, directly induces oocyte maturation.

INTRODUCTION

The meiotic resumption is thought to be triggered by hormonal substances specific to the animal groups. In the sea cucumber (Holothuroidea; Echinodermata), the full-grown oocyte is arrested in the prophase-I stage of meiosis, and meiotic resumption seems to occur just before spawning. Although a number of means have been reported for induction of spawning or oocyte maturation in sea cucumbers (Ohshima, 1925; Inaba, 1937; Colwin, 1948; Strathmann and Sato, 1969; Ishida, 1979, Maruyama, 1980), the endocrine substances responsible for oocyte maturation and spawning are not yet known.

In starfish (Asteroidea; Echinodermata), a gonad-stimulating substance (GSS), a peptide, of the radial nerve stimulates the gonads to induce oocyte maturation and spawning (Chaet and McConnaughy, 1959; Chaet, 1967; Kanatani, 1964, 1973; Kanatani *et al.*, 1971). GSS acts on the follicle cell in the ovary (Hirai and Kanatani, 1971; Cloud and Schuetz, 1973; Hirai *et al.*, 1973) to produce a secondary substance (Kanatani and Shirai, 1967; Schuetz and Biggers, 1967), identified as 1-MeAde (Kanatani *et al.*, 1969). One-MeAde acts on a receptor site on the oocyte membrane to trigger the meiotic resumption (Kanatani and Hiramoto, 1970; Dorée and Guerrier, 1975; Morisawa and Kanatani, 1978; Ikadai and Kanatani, 1982).

The radial nerve factor, a polypeptide, which induces sperm-shedding of isolated testis fragments, was also found in radial nerves of *Strongylocentrotus purpuratus*

Received 23 March 1984; accepted 18 January 1985.

Abbreviations: GSS, gonad-stimulating substance; GVBD, germinal vesicle breakdown; 1-MeAde, 1-methyladenine; RNE, radial nerve extract; RNH, radial nerve homogenate.

[1] Contribution No. 700 from the Seto Marine Biological Laboratory.

(Echinoidea; Echinodermata) by Cochran and Engelmann (1972, 1976). They showed that the radial nerve factor stimulates ovaries to produce an ovarian factor. This factor also induces gamete-shedding from testicular fragments. In sea urchins, 1-MeAde is also reported to play an important role in oocyte maturation and spawning (Kanatani, 1974).

Attempts to demonstrate maturation-inducing activity from the radial nerve of sea cucumbers have been unsuccessful (Noumura and Kanatani, 1962). Several investigators have examined effects of 1-MeAde and starfish GSS on sea cucumber oocyte maturation (Strathmann and Sato, 1969; Stevens, 1970; Hufty and Schroeder, 1974; Ikegami *et al.*, 1976; Kishimoto and Kanatani, 1980; Maruyama, 1980). However, the endocrine substances directly concerned with maturation of sea cucumber oocytes remain unknown, and accumulating evidence suggests that sea cucumber oocyte maturation is controlled by substances other than 1-MeAde. On the other hand, presence of a common mechanism in oocyte maturation is suggested by successful induction of oocyte maturation with such disulfide-reducing agents as dithiothreitol in both sea cucumbers (Maruyama, 1980; Kishimoto and Kanatani, 1980) and starfishes (Kishimoto and Kanatani, 1973). It was also shown that injection of cytoplasm from maturating starfish oocytes, containing maturation-promoting factor, into immature sea cucumber oocytes induced germinal vesicle breakdown (Kishimoto *et al.*, 1982).

The present study reinvestigated effects of the radial nerve of sea cucumbers on oocyte maturation. Water-extracts of radial nerves of sea cucumbers were used. Maturation-inducing activity was assayed with ovarian oocytes in a piece of isolated ovaries, isolated oocytes with the follicle cells, and isolated oocytes deprived of the follicle cells. The presence of a maturation-inducing factor in the radial nerve, its action site, and presence of a secondary factor are examined in this paper.

Materials and Methods

Sea cucumbers (*Holothuria leucospilota, Holothuria pervicax, Holothuria moebi, Holothuria pardalis,* and *Stichopus japonicus*) were collected from June through August (1982 and 1983) near the Seto Marine Biological Laboratory. Except for *S. japonicus,* their gonads were fully developed. Experiments were made in sea water at 27–29°C, the approximate average summer sea water temperature. All tests were intraspecific, *e.g.,* radial nerve preparations and oocytes from the same species, unless otherwise stated.

Preparations of radial nerve homogenate or extract

To obtain the radial nerve of the sea cucumber, the body wall was cut longitudinally with scissors. The radial nerve, located between a pair of bands of the longitudinal muscles, was isolated using a razor and a forceps. This radial nerve preparation contained the radial nerve and its overlying epithelia of water-vascular and coelomic canals. The radial nerves were blotted with filter paper and then weighed. They were used immediately or stored at −20°C until use. Radial nerves were cut into small pieces with scissors, and homogenized, with a glass homogenizer, in de-ionized water for 20 min at 27–29°C. The homogenate was mixed with an equal volume of double strength artificial sea water (modified Herbst's sea water), to make the ionic condition close to the physiological state. This original radial nerve homogenate, usually containing 50 mg wet weight of radial nerves per ml, was used after serial sea water dilution. In some instances, the radial nerve

homogenate (RNH) was centrifuged (26,000 × *g*, 30 min, 4°C). The clear supernatant is designated radial nerve extract (RNE). The 'concentration' of radial nerve component in the extract was defined tentatively by the concentration (mg/ml) of radial nerves in the original homogenate. The radial nerve homogenate or its supernatant were used immediately or stored at −20°C. Storage of the radial nerves at −20°C before or after homogenization did not decrease their activities for up to three months.

The above procedures were also used to obtain homogenates of the other tissues, close to the radial nerve, of the body wall; *i.e.*, longitudinal muscle at the ambulacral zone, and circular muscle and dermis at a middle portion of the interambulacral zone.

Sea water and chemicals

Modified Herbst's sea water (NaCl, 2.6%; KCl, 0.07%; $MgSO_4 \cdot 7H_2O$, 1.2%; $CaCl_2$, 0.11%; $NaHCO_3$, 0.045%) (Motomura, 1938) was used. Ca-free or Ca · Mg-free sea water were prepared, following this formula, with addition of appropriate amounts of NaCl. In some experiments, sodium bicarbonate was replaced by boric acid (0.31%) and pH was adjusted to 8.2–8.3 with NaOH. Pronase (50,000 units, Calbiochem-Behring) was dissolved at 0.1% in sea water. Pronase treatments of RNE were made by incubating RNE (12.5 mg/ml, final concentration) with 0.01% (final) pronase for about 3 hours at 29°C. After heating (85°C, 15 min) to inactivate pronase, the mixture was assayed. In controls, sea water was substituted for the pronase solution. Trypsin (Bovine Pancreas Type III-S, Sigma) was dissolved at 0.1% in sea water. Trypsin treatments were made by incubating RNE (90 mg/ml, final concentration) with 0.01% (final) trypsin at 37°C for 60 min. After addition of soybean trypsin inhibitor (Type I-S, Sigma, 0.06% final conc.), the mixture was assayed. As a control for trypsin treatments, RNE was treated with both 0.01% (final) trypsin and 0.06% (final) soybean trypsin inhibitor at 37°C for 60 min, and then assayed. Dialysis was performed with cellophane tubing (8/32 inch cellophane tubing-seamless, Union Carbide). An aliquot (0.5 ml) of RNE (100 mg/ml) was dialyzed against 0.5 ml of sea water for 17 hours at 4°C, and then assayed. As a control, an aliquot (0.5 ml) of sea water was 'dialyzed' against the same volume of sea water for 17 hours at 4°C, and the 'dialyzate' was assayed. Sephadex G-15 and G-50 (Pharmacia fine chemicals) were swollen in distilled water for 3 hours at 27–28°C, and transferred into columns. The columns were equilibrated with sea water (pH 8.2–8.3).

Bioassay materials

Sea cucumber ovaries with many fully grown oocytes were isolated, immediately washed several times with natural sea water, and then placed in sea water for immediate use.

Ovarian oocytes. Isolated ovaries were cut into pieces, 1 cm long, with fine scissors just before use. After several sea water rinses, the ovarian fragment was transferred into a test solution with forceps. Intact oocytes in such an ovarian fragment are designated 'ovarian oocytes.' In some experiments, an isolated ovary was torn open with two forceps from its cut-ends, longitudinally, and then cut into pieces, 0.5 cm long, with scissors. This 'torn out' ovarian fragment was also used as assay material.

Isolated oocytes with intact follicle cells. Oocytes were squeezed out from freshly isolated ovaries with two forceps. They were immediately washed ten times with

ten volumes of sea water by a hand-centrifuge to avoid sporadic occurrence of the oocyte maturation. The washed oocytes were stored in ten volumes of sea water. Among these washed oocytes, those with intact follicle cell-coats were isolated individually with a micropipette and used as assay materials.

Oocytes deprived of follicle cells. The washed oocytes were treated with ten volumes of Ca-free or Ca · Mg-free sea water two or three times, each for 10–20 min. Follicle cell-free oocytes were isolated individually with a micropipette and used as assay materials.

To determine maturation-inducing activity, about 180 μl of a test solution was placed in a plastic dish. To this drop, one piece of an ovarian fragment, about 20 oocytes with the follicle cells, or about 20 oocytes deprived of the follicle cells, were transferred. Percentage of germinal vesicle breakdown was scored one hour later. In typical experiments using the ovarian fragment, two groups of oocytes (those remaining within the fragment and those extruded from it) were observed separately; the former was observed soon after being squeezed out from the fragment with two forceps. The maturation-inducing activity of the radial nerve of a sea cucumber was assayed by using oocytes of the same species unless otherwise stated.

Preparation of the follicle cell suspension

About 10^6 oocytes were squeezed out of freshly isolated ovaries of *H. leucospilota,* with two forceps, into 40 ml of sea water or Ca · Mg-free sea water. The follicle-oocyte complexes were pipetted several times to enhance detachment of the follicle cells. About 20 min later, the number of oocytes and the percentage of follicle cell-free oocytes were determined. Usually 70–80% of the oocytes were follicle cell-free. Oocytes in the suspension were removed by using a hand-centrifuge twice. The supernatant, virtually free of oocytes or ovarian fragment contamination, was centrifuged at 1500 $\times$ *g* for 15 min to collect pellets of follicle cells. The follicle cells were used after serial sea water dilutions. The density of follicle cells in the suspension was expressed in terms of the number of follicle cell-coats per unit volume, calculated on the basis of the frequency of follicle cell-free oocytes in the original suspension. This calculation may involve a certain degree of over-estimation, since some oocytes were follicle cell-free when they were squeezed from the ovaries.

Results

Presence of maturation-inducing factor in radial nerves

Tissue-homogenates containing radial nerves successfully induced oocyte maturation and 'spawning' in isolated ovarian fragments of *Holothuria leucospilota* and *Holothuria pervicax* (Figs. 1B, C). In *H. leucospilota,* ovarian fragments incubated with radial nerve homogenates (10 mg/ml) began to extrude follicle cell-free oocytes from their cut-ends after a rather constant time-lag of 14 min (n = 4) at 28–29°C. These extruded oocytes matured subsequently; the germinal vesicle began to migrate to the region of the micropyle process at 15 min (after the start of RNH treatment), and after attaching to the region germinal vesicle breakdown (GVBD) occurred at 18–20 min (Maruyama, 1980, 1981). Polar bodies formed from the micropyle process. On insemination, the oocytes began normal development (Fig. 1F). By contrast, control ovarian fragments in sea water did not show such an extrusion of oocytes (Fig. 1A), and the oocytes remaining in the ovary did not mature even after several hours in sea water. These events with a similar time-course were also observed in *H. pervicax* when the ovarian fragments were incubated with the radial nerve homogenate.

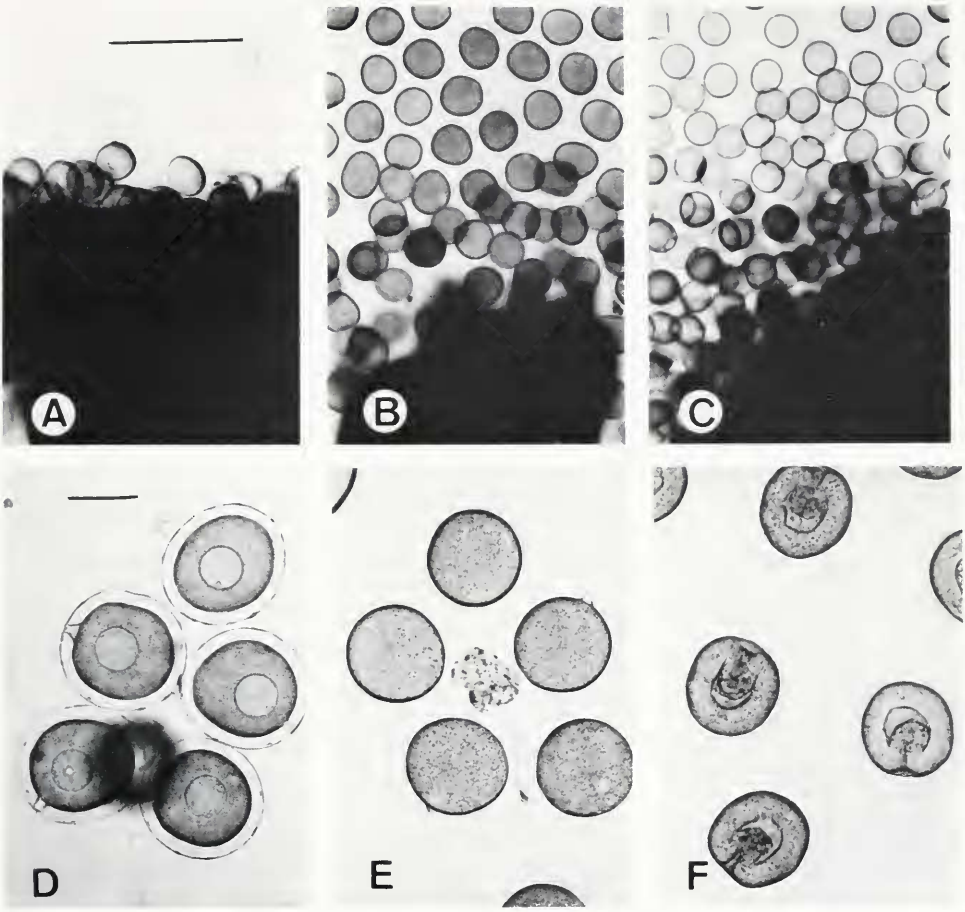

FIGURE 1. Effects of radial nerve homogenates on ovarian fragments and on oocytes with follicle cells. A: control ovarian fragments after 50 min in sea water. *H. leucospilota.* The bar (500 μm) is common to A, B, and C. B: mature oocytes extruded from a cut-end of an ovarian fragment incubated with RNH (10 mg/ml) for 30 min. *H. leucospilota.* C: same as B. *H. pervicax.* D: isolated oocytes with follicle cells in sea water (control). *H. pervicax.* The bar (100 μm) is common to D, E, and F. E: isolated oocytes with follicle cells, incubated with RNE (14 mg/ml) for 40 min. *H. pervicax.* The germinal vesicle is broken down after migrating to the micropyle process. Follicle cells are detached from the oocytes to form a large cell mass (center). F: early gastrulae with invaginating archenteron (16 hours post-fertilization) from oocytes which have been induced to mature by RNH and inseminated 70 min later. The largest optical sections through the main axes of embryos (not compressed) were photographed. *H. leucospilota.*

It is reasonable to regard such an extrusion of oocytes as 'spawning' by an ovarian fragment, because it occurs only after a time-lag following stimulation and most (usually 100%) of the extruded oocytes mature. However, many oocytes still remained in the ovarian fragment after one hour of incubation. Therefore, in experiments using ovarian fragments as assay materials, both oocytes extruded from the fragment and those remaining within the fragment were observed separately, if necessary.

Figure 2 shows GVBD response of ovarian oocytes incubated with various concentrations of radial nerve homogenates in *H. leucospilota.* Threshold concentrations of radial nerve homogenates for inducing spawning lie at 0.5–1.0 mg/ml.

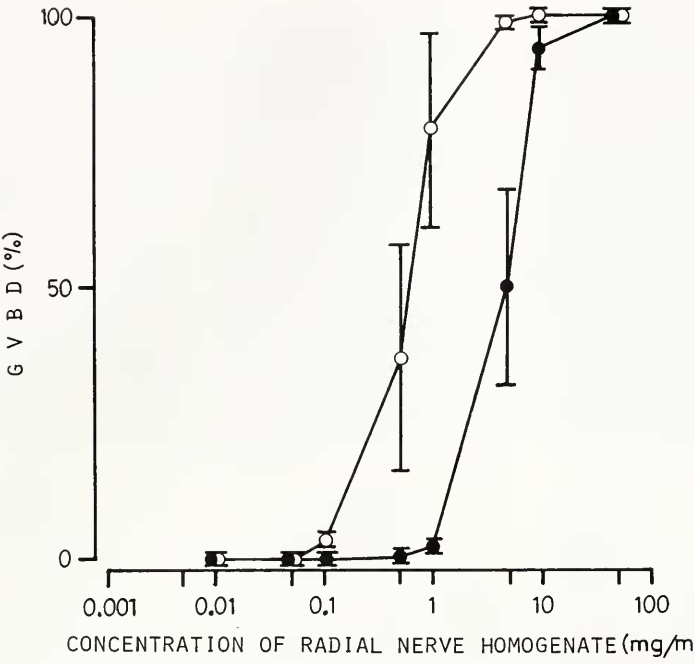

FIGURE 2. Effects of radial nerve homogenate on induction of oocyte maturation in ovarian fragments of *H. leucospilota*. Oocytes extruded from the ovarian fragment (open circles) and those remaining within the ovarian fragment (closed circles) were observed simultaneously. Each point represents mean ± SE of five experiments.

In subthreshold concentrations, a low percentage of oocytes located at the cut-ends of the fragment showed GVBD. There was a range (1–5 mg/ml) of concentrations of radial nerve homogenates where spawning (and subsequent maturation of the spawned oocytes) occurs but GVBD rates of oocytes remaining in the fragment are very low (Fig. 2). The curve indicates that a 10-fold higher concentration is required for GVBD in unextruded oocytes. Nearly all the oocytes remaining in ovarian fragments were induced to mature at a concentration of 5–10 mg/ml. Similar results were observed in other sea cucumbers (Fig. 3A, Table II). A very high concentration (400 mg/ml) of radial nerve homogenate from *H. leucospilota* also successfully induced maturation of all oocytes and spawning in ovarian fragments of *H. leucospilota* and *H. pardalis*.

These results show that the radial nerve has a factor(s) responsible for spawning and oocyte maturation.

Radial nerve homogenates (or extracts) were then applied to isolated oocytes with or without follicle cells (see Figs. 1D, 5A). In *H. leucospilota*, most (83 ± 8%, n = 3) of the isolated oocytes with follicle cells were induced to mature by 1 or 5 mg/ml of radial nerve homogenate. By contrast, none of follicle cell-free oocytes were induced to mature by the homogenate at a wide range of concentration (0.01–50 mg/ml) (n = 3) (*c.f.*, Fig. 5D). In control sea water, no GVBD was observed in oocytes with or without follicle cells. In *H. pervicax* also, radial nerve homogenates at 5 mg/ml induced maturation in most (99 ± 2%, n = 2) isolated oocytes with follicle cells (Figs. 1D, E), but ineffective to follicle cell-free oocytes. In control sea water, no GVBD was observed in oocytes with or without follicle cells. Figure 3B

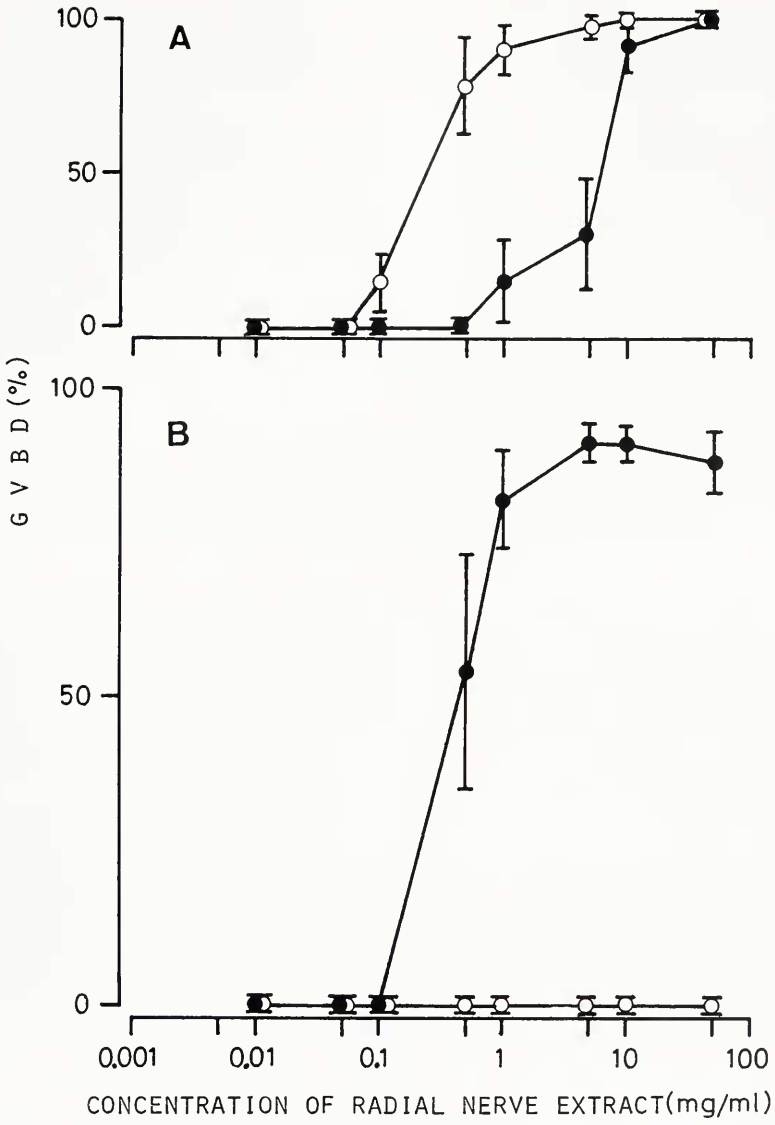

FIGURE 3. Effects of radial nerve extract on induction of maturation in *H. pervicax*. (A) GVBD in oocytes in ovarian fragments. Open circles: oocytes extruded from ovarian fragments. Closed circles: oocytes remaining within ovarian fragments. Each point is mean ± SE of seven experiments. (B) GVBD in isolated oocytes with follicle cells (closed circles) or without follicle cells (open circles). Each point is mean ± SE of three experiments.

shows dose-dependence of GVBD response in isolated oocytes of *H. pervicax*. Sensitivity of isolated oocytes with follicle cells (closed circles in Fig. 3B) is nearly equal to that of the ovarian fragment assessed by extruded oocytes (open circles in Fig. 3A). Also, the threshold concentration of radial nerve extract for inducing maturation of oocytes with follicle cells, attaching to a piece of 'torn-out' ovarian walls, was nearly equal to that in isolated oocytes with follicle cells (data not shown).

These results in *H. leucospilota* and *H. pervicax* show that a maturation-inducing factor in the radial nerve acts on the isolated oocyte with follicle cells, but does not act directly on the follicle cell-free oocyte.

Maturation-inducing activity in tissues adjacent to radial nerves

To examine for maturation-inducing activity in body wall tissues other than the radial nerve, tissue fragments of the interambulacral zone, muscle bands of the ambulacral zone, and radial nerve tissues were separately homogenized from three *H. leucospilota* individuals, and applied to ovarian fragments at a concentration of 5 mg/ml. The frequencies of maturation in oocytes are shown in Table I. Only the radial nerve homogenate exhibited high activity for inducing oocyte maturation and spawning; other tissue homogenates showed low spawning-inducing and maturation-inducing activity even at a higher concentration (50 mg/ml).

These results exclude the possibility of uniform distribution of the maturation-inducing activity all over the body wall, and suggest that the radial nerve is the predominant source of the maturation-inducing activity. Therefore the factor responsible for the activity may be called 'radial nerve factor.' It is possible that the oocyte maturation *in vivo* is due to the factor in the radial nerve.

Common occurrence of radial nerve factor in sea cucumbers

Species-specificity of the radial nerve factor in five species of the order Aspidochirotida was examined by applying radial nerve homogenates at a wide range (0.01–50 mg/ml) of concentrations to ovarian fragments. One hour later the ovarian fragments were observed for spawning and the frequencies of matured oocytes either spawned or remaining in mid-portions of the fragments. In all combinations examined, spawning and oocyte maturation were induced. Table II shows minimal concentrations of radial nerve homogenates required for induction of spawning and maturation. There were no consistent differences in effectiveness between homotypic and heterotypic combinations. By contrast, none of the follicle cell-free oocytes were induced to mature by a wide range (0.01–50 mg/ml) of concentrations of radial nerve homogenates in all combinations examined among *H. leucospilota, H. pervicax, H. pardalis,* and *H. moebi.*

These results indicate that an active factor in the radial nerve is common among five sea cucumber species.

TABLE I

Maturation-inducing activity in tissues adjacent to radial nerves in H. leucospilota

Source of tissue homogenate	Percentage of GVBD[1]	
	5 mg/ml	50 mg/ml
Tissue at interambulacra	0 ± 0[2]	36 ± 30
Longitudinal muscle	0 ± 0	27 ± 12
Radial nerve	100 ± 0	100 ± 0

[1] Percentage of GVBD in oocytes extruded from cut-ends of ovarian fragments at the end of 60 min incubation.

[2] Mean ± SE of three experiments. Neither spawning nor maturation of oocytes was observed in control ovarian fragments in sea water.

TABLE II

Cross-effects of radial nerve homogente on oocyte maturation among sea cucumbers

Source of ovarian fragments	Source of radial nerve homogenate				
	H. leucospilota	*H. pervicax*	*H. moebi*	*H. pardalis*	*S. japonicus*
H. leucospilota	+++ (++)[1]	++ (+)	+ (+)	++ (+)	+ (+)
H. pervicax	+++ (++)	+++ (+++)	+++ (++)	+++ (++)	+ (+)
H. moebi	+++ (++)	+++ (+++)	++ (+)	+++ (++)	?
H. pardalis	++ (+)	++ (+)	++ (+)	++ (+)	?

[1] Symbols indicate minimal concentrations of radial nerve homogenates effective for either spawning (and subsequent maturation) or maturation of oocytes unextruded from the ovarian fragment. The latter is shown in parentheses. +++, 0.1–1 mg/ml; ++, 1–10 mg/ml; +, 10–50 mg/ml; ?, no test.

In *H. leucospilota* and *H. pervicax* radial nerve homogenates from both female and male individuals were similarly effective for inducing the spawning and oocyte maturation, suggesting no sexual differences of the radial nerve factor.

Chemical nature of the radial nerve factor

The chemical nature was examined primarily using radial nerve extract from *H. leucospilota* (Table III). Heating (92°C for 15 min) did not inactivate the maturation-inducing activity of the radial nerve extract. When radial nerve extract was dialyzed against an aliquot of sea water through cellophane tubing, the activity was found in the dialyzate. Pronase (0.01%) and trypsin (0.01%) inactivated the maturation-inducing activity. Similar results were obtained in radial nerve extracts of *H. pervicax*. Such heat-stable, dialyzable, and protease-sensitive properties suggest that the radial nerve factor is a peptide.

The radial nerve factor was separated by gel-filtration. Radial nerve extract of *H. leucospilota* was fractionated on Sephadex G-15 and G-50 columns (Fig. 4). Its elution patterns were simultaneously monitored with 280 nm absorption. An aliquot (180 μl) of each fraction was assayed with an ovarian fragment. In a Sephadex G-15 column, the activity was eluted at the void volume of the column. In a Sephadex G-50 column, the activity was retarded but eluted apparently as a single peak (fraction No. 11, 12, 13, and 14) just before the column volume. Similar results

TABLE III

Effects of heating, dialysis, or protease-treatments of radial nerve extract in H. leucospilota

Treatments	%GVBD[1]	
	Experiments[2]	Controls[2]
Heating (92°C for 15 min)	98%	100%
Dialysis through cellophane tubing	94%	8%
0.01% pronase	0%	95%
0.01% trypsin	0%	100%

[1] Ovarian fragments were used for assay. The GVBD rate was obtained from all the oocytes of an ovarian fragment incubated with a test solution.

[2] See Materials and Methods.

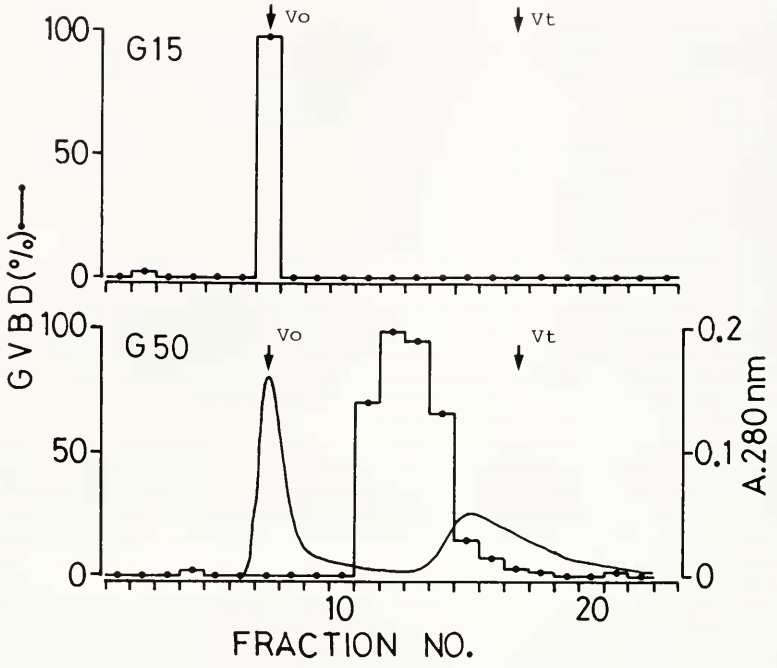

FIGURE 4. Gel-filtration of radial nerve extracts from *H. leucospilota*. Two or three ml of RNE (100 mg per 1 ml of sea water) were placed in Sephadex G-15 columns (1.6 × 42 cm) and Sephadex G-50 columns (1.6 × 42 cm), respectively. Sea water (pH 8.2–8.3) was used as eluant (30 ml/h), and fraction size was 5 ml. Each fraction was assayed with an ovarian fragment, and the GVBD rate was obtained from all the oocytes of the fragment. The elution pattern was simultaneously monitored with 280 nm absorption, and its elution pattern through the G-50 column was shown in a curved-line. The scale of 280 nm absorption is shown in the ordinate at the right side of the figure. Vo, void volume. Vt, column volume or total volume of the packed bed volume.

were obtained in radial nerve extracts of *H. pervicax*. Fractionation range in molecular weight of Sephadex G-50 is 1500–30,000 d ('Sephadex gel-filtration in theory and practice' from Pharmacia Fine Chemicals). The extrusion limit in Sephadex G-15 is reported as 1500 d. Therefore, the molecular weight of the radial nerve factor appears to be larger than 1500 but considerably smaller than 30,000. This implies that the radial nerve factor is a peptide with molecular weight of several thousands' daltons.

Presence of a secondary factor for oocyte maturation

Ovaries or testes (2–3 g, wet weight) of *H. leucospilota* were separately incubated with 1 ml of radial nerve homogenate (50 or 100 mg/ml) for 2 or 3 hours. After removing gonads and spawned oocytes (or sperm in males) by low-speed centrifugation, the incubation mixtures were centrifuged at 26,000 × *g* or 10,000 × *g* for 30 min at 4°C to remove cells or debris, and the resulting supernatants were assayed with follicle cell-free oocytes. The supernatant induced the maturation of follicle cell-free oocytes (Fig. 5). As shown in Table IV, ovaries or testes, in the presence of radial nerves, produce a factor which induces the maturation of the follicle cell-free oocyte. Identical results were obtained by using *H. pervicax*. The controls did not show the maturation-inducing activity (Table IV, Fig. 5D).

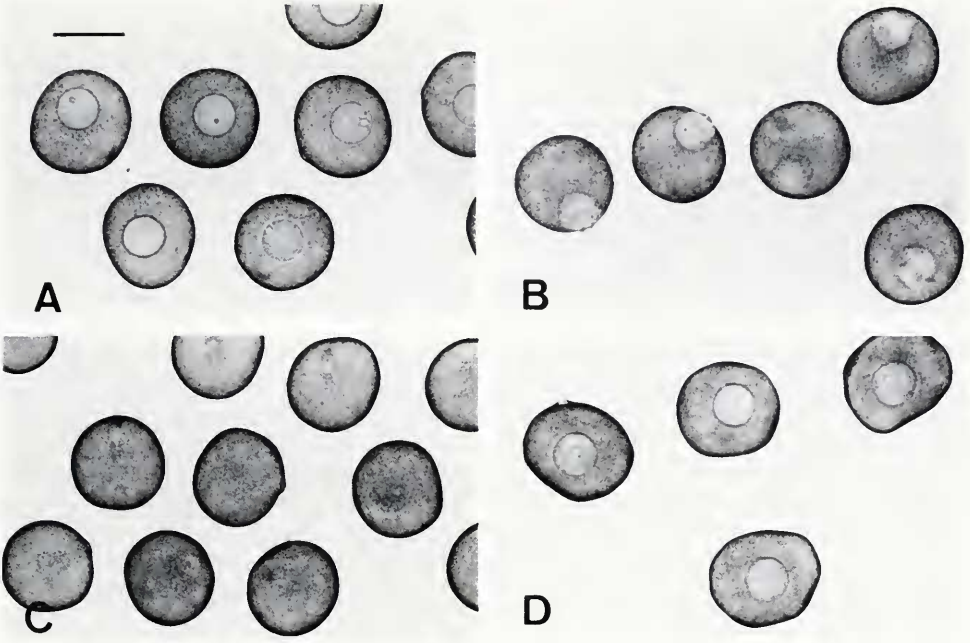

FIGURE 5. Effects on the follicle cell-free oocytes of incubation mixtures of ovaries and radial nerve homogenate in *H. leucospilota*. Ovaries (2–3 g) were incubated with 1 ml of RNE (100 mg/ml) for 2 or 3 hours. The supernatant of the mixture was applied to follicle cell-free oocytes. Water temperature was 28°C. Bar: 100 μm. A: 0 min. B: 20 min after incubation with the supernatant. GVBD just occurs at the micropyle process. C: 110 min. Two polar bodies formed. D: follicle cell-free oocytes incubated with RNH (50 mg/ml) for 60 min.

These results show that a secondary factor which induces maturation in the follicle cell-free oocyte is produced by ovaries or testes incubated with the radial nerve factor.

TABLE IV

Production of a secondary factor by ovaries or testes incubated with radial nerve homogenate (RNH) or extract (RNE)

	No. of experiments	%GVBD of oocytes in serially diluted media				
		1/1	1/2	1/4	1/8	1/16
H. leucospilota						
Ovaries + RNH (50 mg/ml)[1]	4	88 ± 13[2]	24 ± 41	0 ± 0	–	–
Ovaries + RNH (100 mg/ml)	3	98 ± 2	55 ± 34	21 ± 30	0 ± 0	0 ± 0
Ovaries + sea water	3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
RNH (100 mg/ml)	3	2 ± 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Testes + RNH (100 mg/ml)	2	95 ± 0	50 ± 21	3 ± 3	–	–
Testes + sea water	2	0 ± 0	0 ± 0	0 ± 0	–	–
RNH (100 mg/ml)	2	8 ± 2	3 ± 2	3 ± 2	–	–
H. pervicax						
Ovaries + RNE (50 mg/ml)	4	96 ± 3	63 ± 37	12 ± 8	2 ± 3	0 ± 0

[1] The supernatant of the incubation mixture was serially diluted with sea water and assayed with the follicle cell-free oocyte from the same species.

[2] Mean ± SD of GVBD oocytes observed 1 hour later.

Production of a secondary factor by follicle cells

Results in the foregoing sections suggest that follicle cells are the action site of the radial nerve factor, as in the case of starfishes. Follicle cell suspensions were prepared from oocyte-follicle complexes squeezed out from freshly isolated ovaries in *H. leucospilota* (see Materials and Methods). Various densities of follicle cell suspensions were prepared after serial sea water dilutions, and to each suspension the radial nerve homogenate was added at the final concentration of 17 mg/ml. Two or three hours later, an aliquot (180 μl) of each suspension was withdrawn and examined for maturation-inducing activity on follicle cell-free oocytes of *H. leucospilota*. The follicle cell-free oocytes matured with increasing frequency as the density of the follicle cells increased (Fig. 6). Nearly 100% GVBD was obtained with suspensions of 10^{5-6} follicle cell-coats per ml. In control incubations (no follicle cell or radial nerve), the maturation-inducing activity was rarely detected. Both sea water and Ca · Mg-free sea water isolated follicle cells produce maturation-inducing activity (Fig. 6).

These results show that the radial nerve factor acts on the follicle cell to produce a secondary factor, which directly induces maturation of the oocyte.

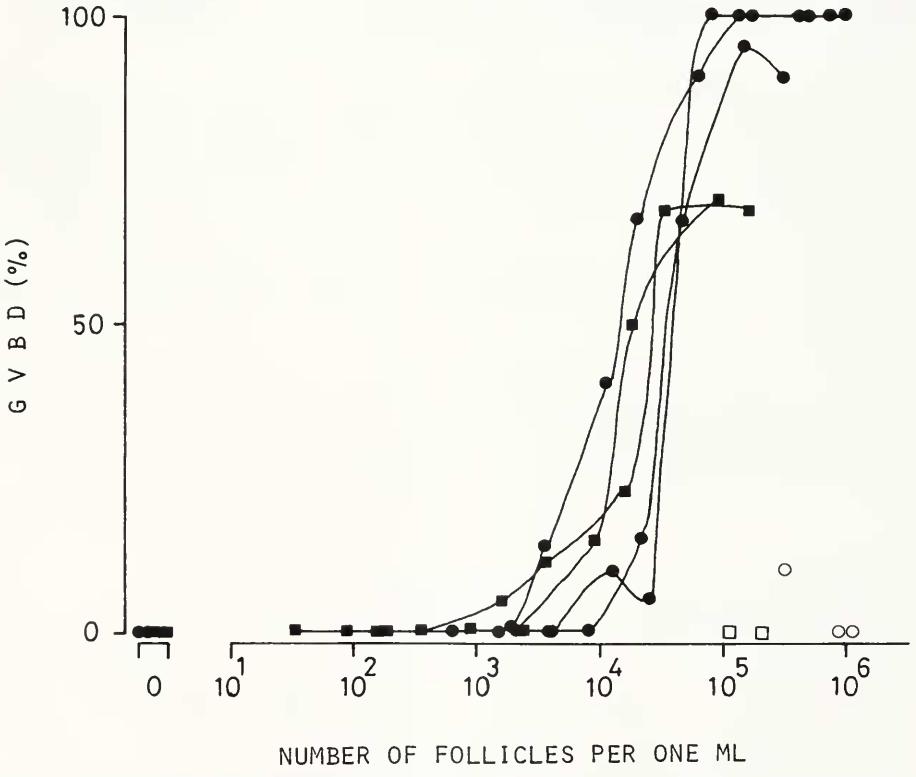

FIGURE 6. Production of a secondary factor by isolated follicle cells incubated with radial nerve homogenate in *H. leucospilota*. The assay was made by the follicle cell-free oocyte. Closed symbols: follicle cell suspensions incubated with RNH (17 mg/ml at the final concentration). Open symbols: original follicle cell suspensions without RNH. Two different media for follicle cell isolation, sea water (squares), and Ca · Mg-free sea water (circles), gave similar results.

Discussion

This study demonstrates a radial nerve factor in five sea cucumber species of the order Aspidochirotida. The factor is apparently a peptide of a low molecular weight (several thousands' daltons) and is common to sea cucumbers. The radial nerve factor acts on oocytes with follicle cells, but does not act directly on an oocyte. This radial nerve factor of the sea cucumber has characteristics similar to radial nerve factor, gonad-stimulating substance (GSS), of starfishes (Kanatani, 1973) and sea urchins (Cochran and Engelmann, 1972, 1976), suggesting that these substances are closely related molecules. It remains unclear what types of cells in the radial nerve tissue contain or secrete the radial nerve factor and whether the factor is produced in tissues other than the radial nerve. Further studies are necessary for identification of the secretory cells and transport route of the radial nerve factor.

This study also demonstrated the presence of a secondary factor which is produced by follicle cells and directly acts on oocyte to mature. These results show that the oocyte maturation of the sea cucumber is regulated via follicle cells. In starfishes, such a secondary factor was identified as 1-MeAde (Kanatani *et al.*, 1969), which is a non-species specific maturation-inducing substance in starfishes (Kanatani, 1973; Kanatani and Nagahama, 1983). However, 1-MeAde has been shown to be ineffective for oocyte maturation of the sea cucumber *H. leucospitota* (Maruyama, 1980, unpub. data). Ineffectiveness of 1-MeAde for sea cucumber oocyte maturation was also reported by Stevens (1970), Ikegami *et al.* (1976), and Kishimoto and Kanatani (1980). The secondary factor of the sea cucumber should be a substance other than 1-MeAde. Purification and identification of the secondary factor in sea cucumbers remains to be done.

The ovarian fragments incubated with the radial nerve homogenate retained a considerable number of immature oocytes even after spawning many oocytes, and a higher concentration of the radial nerve homogenate was required for the maturation of the remaining oocytes. This might suggest the presence of some inhibitors within ovaries against the action of the radial nerve factor. Spawning inhibitors have been reported in starfish ovaries (Ikegami *et al.*, 1967; Ikegami, 1976).

The accumulated evidence shows the neurosecretory mechanism of oocyte maturation or gamete-shedding in three out of five classes of Echinodermata. In addition, a regulatory (inhibitory) system to such a control is now found in sea cucumbers as well as starfishes. Demonstration of similar neurosecretory mechanism in the other classes of Echinodermata is awaited.

Acknowledgments

I want to thank Professor E. Harada and other members of the Seto Marine Biological Laboratory for facilitating my work there. I am especially grateful to Professor M. Yoneda for critically reading the manuscript.

Literature Cited

Chaet, A. B. 1967. Gamete release and shedding substance of sea-stars. *Symp. Zool. Soc. Lond.* **20:** 13–24.

Chaet, A. B., and R. A. McConnaughy. 1959. Physiologic activity of nerve extracts. *Biol. Bull.* **117:** 407–408.

Cloud, J., and A. W. Schuetz. 1973. Spontaneous maturation of starfish oocytes: role of follicle cells and calcium ions. *Exp. Cell Res.* **79:** 446–450.

Cochran, R. C., and F. Engelmann. 1972. Echinoid spawning induced by a radial nerve factor. *Science* **178:** 423–424.

Cochran, R. C., and F. Engelmann. 1976. Characterization of spawn-inducing factors in the sea urchin, *Strongylocentrotus purpuratus*. *Gen. Comp. Endocrinol.* **30:** 189–197.

COLWIN, L. H. 1948. Note on the spawning of the holothurian, *Thyone briareus* (Lesueur). *Biol. Bull.* **95:** 296–306.

DORÉE, M., AND GUERRIER. 1975. Site of action of 1-methyladenine in inducing oocyte maturation in starfishes: kinetical evidences for receptors localized on the cell membrane. *Exp. Cell Res.* **91:** 296–300.

HIRAI, S., AND H. KANATANI. 1971. Site of production of meiosis-inducing substance in ovary of starfish. *Exp. Cell Res.* **67:** 224–227.

HIRAI, S., K. CHIDA, AND H. KANATANI. 1973. Role of follicle cells in maturation of starfish oocytes. *Dev. Growth Differ.* **15:** 21–31.

HUFTY, H. M., AND P. C. SCHROEDER. 1974. A hormonally active substance produced by the ovary of the holothurian *Parastichopus californicus*. *Gen. Comp. Endocrinol.* **23:** 348–351.

IKADAI, H., AND H. KANATANI. 1982. Regeneration of oocyte-surface factor responsible for 1-methyladenine-induced oocyte maturation in starfish. *Biomed. Res.* **3:** 378–383.

IKEGAMI, S. 1976. Role of asterosaponin A in starfish spawning induced by gonad-stimulating substance and 1-methyladenine. *J. Exp. Zool.* **198:** 359–366.

IKEGAMI, S., S. TAMURA, AND H. KANATANI. 1967. Starfish gonad: action and chemical identification of spawning inhibitor. *Science* **158:** 1052–1053.

IKEGAMI, S., H. KANATANI, AND S. S. KOIDE. 1976. Gamete-release by 1-methyladenine *in vitro* in the sea cucumber, *Leptosynapta inhaerens*. *Biol. Bull.* **150:** 402–410.

INABA, D. 1937. On artificial insemination in sea cucumbers (in Japanese). *J. Fish.* **32:** 241–246.

ISHIDA, M. 1979. Studies on seed production of the sea cucumber, *Stichopus japonicus* (in Japanese). Pp. 1–17 in *Report of Buzen Fisheries Experiment Station*, the issue of the year 1977, Fukuoka.

KANATANI, H. 1964. Spawning of starfish: action of gamete-shedding substance obtained from radial nerves. *Science* **146:** 1177–1179.

KANATANI, H. 1973. Maturation-inducing substance in starfishes. *Int. Rev. Cytol.* **35:** 253–298.

KANATANI, H. 1974. Presence of 1-methyladenine in sea urchin gonad and its relation to oocyte maturation. *Dev. Growth Differ.* **16:** 159–170.

KANATANI, H., AND H. SHIRAI. 1967. *In vitro* production of meiosis inducing substance by radial nerve extract in ovary of starfish. *Nature* **216:** 284–286.

KANATANI, H., H. SHIRAI, K. NAKANISHI, AND T. KUROKAWA. 1969. Isolation and identification of meiosis inducing substance in starfish *Asterias amurensis*. *Nature* **221:** 273–274.

KANATANI, H., AND Y. HIRAMOTO. 1970. Site of action of 1-methyladenine in inducing oocyte maturation in starfish. *Exp. Cell Res.* **61:** 280–284.

KANATANI, H., S. IKEGAMI, H. SHIRAI, H. OIDE, AND S. TAMURA. 1971. Purification of gonad-stimulating substance obtained from radial nerves of the starfish *Asterias amurensis*. *Dev. Growth Differ.* **13:** 151–164.

KANATANI, H., AND Y. NAGAHAMA. 1983. Echinodermata. Pp. 611–654 in *Reproductive Biology of Invertebrates*, Vol. 1, K. G. and R. G. Adiyodi, ed. John Wiley and Sons, Chichester.

KISHIMOTO, T., AND H. KANATANI. 1973. Induction of starfish oocyte maturation by disulfide-reducing agents. *Exp. Cell Res.* **82:** 296–302.

KISHIMOTO, T., AND H. KANATANI. 1980. Induction of oocyte maturation by disulfide-reducing agent in the sea cucumber, *Stichopus japonicus*. *Dev. Growth Differ.* **22:** 163–167.

KISHIMOTO, T., R. KURIYAMA, H. KONDO, AND H. KANATANI. 1982. Generality of the action of various maturation-promoting factors. *Exp. Cell Res.* **137:** 121–126.

MARUYAMA, Y. K. 1980. Artificial induction of oocyte maturation and development in the sea cucumbers, *Holothuria leucospilota* and *Holothuria pardalis*. *Biol. Bull.* **158:** 339–348.

MARUYAMA, Y. K. 1981. Precocious breakdown of the germinal vesicle induces parthenogenetic development in sea cucumbers. *Biol. Bull.* **161:** 382–391.

MORISAWA, M., AND H. KANATANI. 1978. Oocyte-surface factor responsible for 1-methyladenine-induced oocyte maturation in starfish. *Gamete Res.* **1:** 157–164.

MOTOMURA, I. 1938. Effects of some salt solutions on the parthenogenetic membrane formation of sea urchin eggs. *Sci. Rep. Tōhoku Univ. Ser. IV (Biol.)* **13:** 85–88.

NOUMURA, T., AND H. KANATANI. 1962. Induction of spawning by radial nerve extracts in some starfishes. *J. Fac. Sci. Univ. Tokyo, Sec. IV.* **9:** 397–402.

OHSHIMA, H. 1925. Notes on the development of the sea-cucumber, *Thyone briareus*. *Science* **61:** 420–422.

SCHUETZ, A. W., AND J. D. BIGGERS. 1967. Regulation of germinal vesicle breakdown in starfish oocytes. *Exp. Cell Res.* **46:** 624–628.

STEVENS, M. 1970. Procedures for induction of spawning and meiotic maturation of starfish oocytes by treatment with 1-methyladenine. *Exp. Cell Res.* **59:** 482–484.

STRATHMANN, R. R., AND H. SATO. 1969. Increased germinal vesicle breakdown in oocytes of the sea cucumber *Parastichopus californicus* induced by starfish radial nerve extract. *Exp. Cell Res.* **54:** 127–129.